CULTURAL METHODS FOR THE GONOCOCCUS *

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In connection with a serologic study [1] of the gonococcus it was necessary for us to devise methods and mediums whereby this organism might be isolated readily from gonorrheal infections, and also a large collection of strains might be maintained with a minimal risk of any of them being lost. The difficulties inherent in the cultivation of the gonococcus are evidenced by the great variety of mediums and procedures which have been recommended for this purpose, especially within recent years. At present there seems to be no consensus of opinion as regards the best methods to use. In the following section certain simple mediums are described which have given good results consistently in connection with the various purposes specified. It may be remarked that through the use of a sterilizable medium of proper reaction hardly greater difficulty has been encountered in maintaining a large collection of gonococcus strains than would be associated with carrying a like number of typhoid strains.

In another article [2] are reported the results obtained through the application of these methods to the isolation of gonococcus strains from cases of mild chronic gonorrhea in women, together with a comparative study of the relative value of the cultural, complement-fixation and smear methods of diagnosis of such cases.

MEDIUMS

As a matter of convenience, the mode of preparation of certain of the mediums employed in these experiments will be described here, leaving the discussion of their uses and particular advantages to subsequent sections.

A. Ascitic-veal-urine-glycerol-agar. This plating medium, especially when combined with the dye, iodin-green, was found effective in the isolation of the gonococcus. The Thalmann [3] method of preparation has been followed to some extent, but the formula has been materially modified.

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[1] Jour. Immunol., 1922.

[2] Jour. Infect. Dis., 1922, 31, p. 148.

[3] Centralbl. f. Bakteriol., I, O., 1900, 27, p. 828.

Place 1,250 gm. of fresh, chopped, fat-free veal and 2 liters of distilled water in a pot and bring slowly to a boil, allowing it to simmer for 20 minutes, with occasional stirring. Strain through cotton flannel, cool and remove the fat. Place in a double boiler over a saturated brine bath and raise the temperature to about 60 C. Add 20 gm. of peptone (Difco used), 40 c c normal, fresh urine, 10 gm. NaCl, 40 c c glycerol and 36 gm. flaked agar. Allow this to boil for 45 minutes and then adjust the reaction to P_H 6.9, using 10% sodium carbonate, and boil for 30 minutes longer. Remove from the brine bath; make up loss from evaporation to 2 liters with distilled water. Filter through canton flannel and tube in 10 c c amounts. Autoclave at about 12 lbs. pressure for 10 minutes.

In preparing the plates, 5 c c of ascitic fluid, free from bile, and 0.5 c c of a 1:3,000 dilution of iodin-green (Grübler) are added to each tube of melted medium, just before pouring. The final reaction is generally about P_H 7.2. It should not be more alkaline than this.

If to be used in slants, the amount of agar is increased to 40 gm.

The following mediums contain a growth accessory principle. They are modifications of the "hormone" mediums, described by Huntoon,[4] and have been found very useful for the purposes specified. As beef heart constitutes the meat element, they may be prepared at slight expense.

B. Ascitic, salt-free, 1.5% peptone, "vitamine" agar. This medium has been employed in connection with the isolation of the gonococcus.

Five hundred gm. of fresh, chopped beef heart, free from fat (the beef heart meat should preferably be obtained directly from the slaughter house), one whole egg and 1 liter of distilled water are placed in a double boiler over a free flame and the temperature maintained at 60 C., with constant stirring, for 5 minutes. Fifteen gm. of peptone (Difco) and 18 gm. of flaked agar are now added and the temperature raised until the mixture assumes a brownish color. The medium is then made slightly alkaline to litmus, using a 10% solution of sodium carbonate. It is next placed in a flask, or preferably a coffee pot, and heated at 100 C. in the Arnold steam sterilizer for 1 hour. The clot is then separated from the sides of the receptacle, and it is replaced in the sterilizer for another hour. It may be cleared by centrifuging or by straining through a fine wire mesh and then through glass wool. A clear medium may often be obtained if the meat residue is deposited on the glass wool in a funnel and the fluid portion allowed to percolate through several times. As Huntoon has stipulated, neither cloth, cotton nor any other material with absorptive properties should be used in clarifying the medium. After filtration, the reaction is brought to P_H 6.8. It is then reheated and tubed in 10 c c amounts. It may be sterilized in the autoclave at 12 lbs. pressure for 10 minutes, but fractional sterilization at 100 C. flowing steam is preferable. In preparing the plates, 5 c c of ascitic fluid is added to each tube of melted medium, just before pouring.

A modification of this medium, prepared without ascitic or other serous fluid and which has been found useful in obtaining growths for complement-fixation tests, is described elsewhere.[1]

[4] Jour. Infect. Dis., 1918, 23, p. 169.

C. A semi-solid agar with a growth accessory element (Huntoon). This medium, without the addition of ascitic fluid, has been found useful for maintaining our collection of stock strains. Even the most delicate strains of gonococcus have remained viable for 3 to 4 weeks without replanting, and the majority have lived for periods of 6 to 8 weeks or even longer, if kept at 37 C. The ingredients of this medium are the same as those stated in the formula of Huntoon.

Distilled water	1000 c c
Fresh chopped beef heart	500 gm.
Peptone	10 gm.
NaCl	5 gm.
One whole egg	

The same procedures are followed as in the preparation of medium B, and the final reaction is adjusted to P_H 6.8. This medium is tubed in about 7 cc amounts and is preferably sterilized in the Arnold sterilizer. The method of inoculating and the uses for this medium will be described. If ascitic fluid to the amount of about 1 cc per tube is added, the medium may be employed in connection with primary fishings of gonococcus colonies. It may also be used advantageously in the rejuvenation of delicate strains and in the recovery of old stock strains which have apparently died out.

CONDITIONS FAVORING MAXIMAL GROWTH OF THE GONOCOCCUS

Cole and Lloyd[5] in their analysis of the cultural requirements of the gonococcus, determined that there are three factors of prime importance: first, the concentration of the hydrogen ions, or the reaction; second, the concentration of amino acids; and third, the presence of certain growth stimulating hormones or vitamines. The following paragraphs contain a report of our experiments on the effect of these various factors on the growth of this organism.

Reaction.—Cole and Lloyd reported the mean optimal reaction for growth as P_H 7.6, with a possible range of hydrogen-ion concentration between 6.5 and 8.6. They also stated that with relatively simple (unfavorable) mediums the reaction is of great importance, whereas in mediums containing a growth stimulating substance, a wide reaction range is compatible with good growth. Our findings agree in general with these statements, although our point of optimal growth is nearer neutrality. This lack of conformity as regards the optimal point may be due in part to the use of different standards for determining the H-ion concentration. We have used the colorimeter methods of Clark and Lubs,[6] employing standard buffer solutions prepared according to their directions and which were, in turn, checked with solutions prepared in several other laboratories. They employed, on the other hand, the standards

[5] Jour. Path. & Bacteriol., 1916-17, 21, p. 269.
[6] Jour. Bacteriol., 1917, 2, p. 1.

of Cole and Onslow.[7] Warden,[8] several years ago, emphasized the importance of a slightly acid (+0.7 to 1.5 to phenolphthalein) reaction in mediums for the successful cultivation of the gonococcus. It is also our opinion that most investigators have employed mediums with a somewhat too alkaline reaction. Swartz[9] has recently reported successful growth on an ascitic agar medium with a H-ion range from 6.5 to 8, if this organism is cultivated under conditions of partial oxygen tension. Erickson and Albert,[10] using a beef testicular agar enriched with blood, found that the optimal reaction lay between P_H 7.4 and 7.6.

With our medium A, enriched with ascitic fluid, we have found that good growth may be obtained with a reaction ranging from about P_H 6.6 to 7.4, but if this medium is used without ascitic fluid the range is still more restricted toward the neutral point. With agar medium containing a growth accessory principle, a much greater latitude in reaction is permissible. The semisolid medium C has produced growth within a reaction range of from P_H 5.8 to 8.2, with an optimal zone between 6.4 and 7.7.

In table 1 are given both the degrees of growth at various H- ion concentrations with a stock strain of gonococcus, long under cultivation, and the remarkable period of viability observed in the lots of the medium with the more acid reaction. In this experiment 10 tubes of semisolid vitamine agar (C) of each reaction were seeded with gonococcus strain 33; the tubes were sealed with paraffin and kept at 36-37 C. From time to time replants were made into this medium C, which had been enriched with ascitic fluid, or on ascitic agar slants. With so many of the original cultures to choose from in testing for viability, it was possible to plant either from a hitherto unopened tube or, at least, from an undisturbed part of the original growth in a tube which had been opened before. As table 1 indicates, a tube with an initial reaction of P_H 6.3 still held viable organisms after the lapse of a year. With this particular strain, too, successful growths were obtained for far longer periods from the tubes on the acid side of neutrality than from those on the alkaline. It should be noted, however, that the initial reactions did not remain constant, but that the gonococcus growth caused a gradual change in the direction of alkalinity. On replanting from tubes which had been kept several months, the gonococcus colonies developed very slowly, no growth frequently appearing for 3 or 4 days. Cole and Lloyd[5] have also reported long

[7] Lancet, 1916, 2, p. 9.
[8] Jour. Infect. Dis., 1913, 12, p. 93.
[9] Jour. Urol., 1920, 4, p. 325.
[10] Jour. Infect. Dis., 1922, 30, p. 269.

viability periods for gonococcus when growing in stab culture in their 2% tryptamine blood agar with an H- ion concentration of about 7.6, some of their strains surviving from 67 days to 5½ months. Morax [11] found that ascitic agar stab cultures might retain their viability for 6 months. Warden,[7] using an artificial serum fluid medium, found that successful transplantation could be made after 100 days.

As the gonococcus surviving for one year without replanting was from an old stock strain and probably an unusually hardy one, a number of similar tests were conducted with recently isolated strains (table 2). As may be noted, the reaction of the medium, optimal for growth, was close to the neutral point. As regards the relation of reaction to viability, the tests with these strains yielded rather irregular results but, on the whole, at the periods of replanting a greater num-

TABLE 1

RELATION OF GROWTH TO VARIOUS REACTIONS AND THE RELATIVE PERIODS OF VIABILITY AT VARIOUS REACTIONS. UNENRICHED SEMISOLID MEDIUM C

H-ion Concentration of Medium	Degrees of Growth	Viability Tests: Replants in Weeks After Primary Cultures									
		3	4	8	9	16	22	29	42	46	52
5.4	—										
5.8	++	+	+	+	+	+	+	+	+	+	—
6.3	+++	+	+	+	+	+	+	+	+	+	+
6.8	+++	+	+	+	+	+	+	—	—	—	—
7.4	+++	+	+	+	—	—	—	—	—	—	—
7.8	++	+	—	—	—	—	—	—	—	—	—
8.4	—										

ber of cocci were found viable in the tubes with a slight degree of acidity than in those with the more alkaline reaction. A difference in the character of the growth in relation to the reaction was also noted in connection with all the strains. After about a week's incubation, the growth on the slightly acid to neutral medium became thick, soft and slightly buff-colored, whereas on the alkaline side of neutrality it was thinner, white and generally drier in appearance. These differences were also noted by Cole and Lloyd. They observed, too, that autolysis apparently proceeds more rapidly on mediums more alkaline than that considered the mean optimum. Our observations add confirmation to this conclusion.

As to the relation of the reaction of the medium to viability, the tests with these strains yielded rather irregular results but, on the

[11] Ann. de l'Inst. Pasteur, 1918, 32, p. 471.

whole, at the times of replanting a greater number of cocci were found viable in the slightly acid tubes than in those with the more alkaline reaction.

These periods of viability (table 2), which are by no means the limits for even these delicate strains, contrast strongly with those obtained by others on slants of solid medium. Swartz [10] has reported a viability period of 7 to 10 days on slants of his ascitic agar medium and Cook and Stafford [12] of only 8 days with slants of their enriched

TABLE 2

RELATION OF VARIOUS REACTIONS TO THE GROWTH OF RECENTLY ISOLATED GONOCOCCUS STRAINS AND RELATION OF REACTION TO VIABILITY *

H-ion Concentration of Medium	Relative Degrees of Growth, Gonococcus Strains								Tests for Viability: Colony Development from One Loop					
	28	63	66	67	70	74	75	76	28 30 Days	66 21 Days	70 21 Days	74 45 Days	75 26 Days	76 26 Days
6.0	1	1	1	2	0	0	2	0	Numerous	Fairly numerous	—	—	∞	—
6.4	3	3	2	2	0	1	3	1	∞	0	—	Few	∞	∞
6.8	3	3	2	2	2	1	3	1	0	0	Very numerous	Numerous	Fairly numerous	∞
7.3	3	2	3	2	2	1	2	2	0	0	Very numerous	0	∞	Few
7.7	2	1	2	2	1	1	2	1	0	Fairly numerous	Numerous	Few	Numerous	Numerous
8.0	2	...	1	1	0	0	1	1	Fairly numerous	0	—	—	Fairly numerous	Fairly numerous
8.2	1	1	1	1	0	0	0	0	0	0	—	—	—	—
8.6	...	...	0	0	0	0	...	...	..	—	—	—	—	—

Strains 75, 76: third generation from isolation.
Strain 74: fourth generation from isolation.
Strains 63, 66, 70: fifth generation from isolation.
Strain 28: very delicate strain.
* Primary plants were made on semisolid agar (C) and tubes sealed with paraffin. Replants for viability were made on ascitic agar slants, using one uniform loop of the primary culture.

testicular agar, and no recoverable growth after 3 days with stab cultures. Hermanies,[13] however, using salt-free ascitic agar slants, about neutral to litmus, found that some of his strains would live for weeks or even months without transplanting.

Amino Acids.—Cole and Lloyd have emphasized the important influence of amino acids on the development of the gonococcus, especially in the presence of "growth hormones." Their amino acids were obtained by a tryptic digestion of casein and the product was desig-

[12] Jour. Infect. Dis., 1921, 29, p. 561.
[13] Ibid., 1921, 28, p. 133.

nated "tryptamine." In experiments bearing on this point we have used preparations from liver, casein and beef in which the amino acids constituted from 65 to 75% of the total nitrogen.[14] These products were used in 1% amounts in place of peptone in preparing the semi-solid medium C, and the growth and viability on these mediums were compared with that on the medium prepared with peptone, but otherwise identical. It was not found that these mediums, unusually rich in amino acids, produced a greater or a quicker growth; in fact, with some strains the results were inferior to those obtained with the peptone medium. Accordingly it was concluded that the peptone [15] used, especially if employed in a concentration of 1.5%, contained sufficient amino acids to effect the optimal development of the gonococcus. In fact, M'Leod and Wyon [16] recently reported that high concentrations of amino acids readily inhibit the growth of such organisms as pneumococci, meningococci and hemolytic streptococci.

Growth Stimulating Substances.—A third factor, considered by Cole and Lloyd of primary importance in the cultivation of the gonococcus, are two different growth stimulating substances, present in fresh blood and other materials, which were designated as "growth hormones." The substance of importance in inducing initial growth was considered a derivative of red blood cells and was shown to be readily absorbable by colloidal substances, such as agar and gelatin, and also by materials ordinarily used in clarifying mediums. The second substance, present in animal and plant tissues, was relatively non-absorbable, and was thought to stimulate luxuriant secondary growth. The first substance was considered to be of the same nature as vitamines or hormones because of the ease with which it was absorbed. As the term, vitamine, would seem to be a rather more appropriate designation for the substance stimulating primary growth, it will be employed hereafter.

Huntoon,[4] utilizing these principles of Cole and Lloyd, has described simpler methods for preparing these vitamine mediums. Instead of blood, beef heart or steak was employed with the idea that these tissues would provide sufficient of the growth accessory substances, especially when fortified with a whole egg. We have found mediums of this type useful in our work with the gonococcus, and have followed

[14] Prepared by the Arlington Chemical Co.

[15] Different lots of this peptone have varied somewhat in efficiency as growth producers, but only one sample was encountered which could not be used.

[16] Jour. Path. & Bacteriol., 1921, 24, p. 205.

Huntoon's methods with certain modifications in the adjustment of the reaction, the amount of peptone and the use of salt. Beef hearts have been used entirely as the meat base, and it has been deemed important to obtain them as fresh as possible. In all probability the primary growth stimulating substance is derived mostly from the blood content of the meat and in minor degree from the tissues.

The desirability of limiting the degree and duration of heating in the preparation of mediums of this type was emphasized by Huntoon on the supposition that the growth stimulating substances are to some degree heat labile. Thjotta and Avery,[17] in a careful analysis of the properties of the growth accessory substances essential for the cultivation of the hemophilic bacilli, determined that their V factor, derivable from blood and from yeast and other vegetable cells, was not impaired appreciably by boiling for 10 minutes, but their potency was greatly impaired by exposure in the autoclave to 120 C. for 30 minutes. In the preparation of these vitamine mediums for the gonococcus, it has been necessary to apply a boiling temperature for far longer periods than that specified by Thjotta and Avery as a safe limit. There can be no doubt, however, that the growth stimulating substances for the gonococcus survive this rather prolonged exposure to 100 C. in sufficient amount and degree to influence its development very favorably. That this is the case is shown by the experiments of Cole and Lloyd and also by our own results. The question arose, however, as to whether these growth accessory substances would resist the higher temperature of the autoclave. In order to test the effect of this heat factor a number of the most delicate strains in our collection were seeded on a particular lot of the semisolid vitamine agar (C) the several portions of which were subjected to the various degrees and durations of heating, as shown in table 3. Under column B of this table there is also demonstrated the adsorptive action of flannel and cotton on these accessory growth substances. Thjotta and Avery also found that their V factor, essential to the growth of B. influenzae, was readily adsorbed by bone charcoal.

The results reported in table 3 indicate that the growth stimulating substance in this medium is slightly impaired by the autoclave temperature of 120 C. for 5 minutes and seriously injured by a temperature of 120 C. for 30 minutes, and also by prolonged heating in the Arnold sterilizer. In estimating the degree of injury the results with lots A

[17] Jour. Exper. Med., 1921, 34, p. 97.

and B serve as good comparates; in the former the medium exhibits its maximal efficiency, whereas from the latter the growth stimulating element has been in large degree removed by filtration through absorbing substances.

Moisture.—It has long been realized that the gonococcus will not grow well on solid mediums from which the initial moisture has largely dried out. Only recently, however, has the importance of a moist air in the incubator been appreciated. Jenkins [18] has reported that a moist atmosphere, obtained by placing a large dish of water in the incubator, materially accelerates the growth of the gonococcus. Cook and

TABLE 3

THE EFFECT OF FILTRATION AND OF DIFFERENT DEGREES AND PERIODS OF HEATING ON THE GROWTH STIMULATING PRINCIPLE OF AGAR MEDIUM C *

Gonococcus Strains	A		B		C		D		E	
	3 Days	12 Days	3 Days	12 Days	3 Days	12 Days	3 Days	12 Days	3 Days	12 Days
3	++	++	—	—	—	—	—	—	—	—
40	—	++	—	—	—	—	—	++	—	—
54	++++	++++	—	—	+	++	+++	++++	++	++
69	+	+	—	—	—	—	—	—	—	—
27	+++	+++	—	—	++	+++	+++	+++	+	++
41	++++	++++	++	++++	+	++	++	++++	++	++++
34	++++	++++	++++	++++	++++	++++	++++	++++	++	++++

+, ++, +++, ++++ indicate degrees of growth.

* Different portions of one lot of this semisolid medium treated as follows:

Lot A: Filtered through glass wool and heated at 100 C. in the Arnold Sterilizer for 20 minutes on 3 consecutive days.

Lot B: Filtered twice through flannel and absorbent cotton and sterilized as A.

Lot C: Filtered through glass wool and heated in the Arnold sterilizer for 2 hours on each of 3 consecutive days.

Lot D: Filtered as for C and heated in the autoclave at 120 C. for 5 minutes.

Lot E: Filtered as for C, and heated in the autoclave at 120 C. for 30 minutes.

The H-ion concentrations of these several lots after final sterilization ranged from PH 6.5 to 7.0 (within the zone of optimum growth).

Tubes of these several lots were seeded with one loop each of a saline suspension (about 3 billion strength) of gonococci. Strains 3, 40, 54, 69, 27 were of the delicate type.

Stafford [12] found that the best growths resulted when the culture tubes were placed in closed jars containing water and that placing a pan of water in the incubator was not sufficient. It has been our experience that a large bowl or pan of water (about 10 inches in diameter) provides enough moisture for the ordinary sized incubator. There can be no doubt that the presence of such a moist atmosphere is a matter of prime importance, especially in obtaining primary growth from infected material. It acts as a marked accelerator of growth; colonies appear several hours earlier on the plates and in greater number than

[18] Jour. Bacteriol. & Path., 1921, 24, p. 160.

when incubated without special provision for moisture; in fact, there may be no growth at all in the presence of the ordinary dry air of the incubator.

As regards the physical state of solid medium, we have found that the best results are obtained when it is moderately moist and firm. For slants, after the proper amount of ascitic fluid has been added, the agar content should amount to about 1.5% and for plates rather less than that. Hall [19] has advised a hard, firm surface, free from excessive moisture, for optimal growth, but we believe that a very firm surface, such as is obtained with 2.5% agar, is decidedly unfavorable. Ascitic agar slants should be prepared the day before they are to be used and should generally be allowed to drain for an hour in the incubator before seeding. Culture plates should be poured several hours before they are to be streaked, or, if the medium has its full quota of moisture, may be prepared the day before.

Reduced Oxygen Tension.—Within recent years a number of investigators have advocated a reduced oxygen tension atmosphere as especially favorable for the growth of the gonococcus. This reduction in tension has been effected in various ways; through heating the air in the culture tube and closing tightly with a rubber stopper (Ruediger,[20] Swartz [9]); through a partial exhaustion of the air (Swartz [9]); through exposure to a CO_2 atmosphere (Chapin [21]); through the use of a bacterial culture with oxygen reducing properties, such as B. subtilis (Wherry and Oliver,[22] Herrold,[23] Hermanies [13]). From time to time we have tested these various procedures, but the results have been in no way superior to those obtained on the same medium exposed to moist air of normal pressure. The gonococcus in fact does not behave like an organism with a predilection for reduced oxygen tension. In stab cultures the growth in the stab is, at best, very feeble; in fact, in our semisolid medium it occurs exclusively on and immediately below the surface. It is worthy of note that all the procedures utilized for maintaining a reduced oxygen tension also tend to retain moisture in the medium or in the air, which is in itself an important factor in obtaining the maximal growth for the gonococcus.

[19] Jour. Bacteriol., 1916, 1, p. 343.
[20] Jour. Infect. Dis., 1919, 24, p. 376.
[21] Ibid., 1918, 23, p. 342.
[22] Ibid., 1916, 19, p. 288.
[23] Jour. Am. Med. Assn., 1921, 76, p. 225.

Cook and Stafford [11] and Erickson and Albert [9] have also recently reported that a reduced oxygen tension does not favor the growth of the gonococcus.

THE PRIMARY ISOLATION OF THE GONOCOCCUS

As it was seldom feasible to inoculate the culture plates immediately after the infected material had been obtained from gonorrheal cases, the following method was devised whereby a delay of several hours might elapse with a minimal risk of loss of viability on the part of the gonococci.

One c c portions of a mixture of 2 parts of semisolid "vitamine" agar (C) and 1 part of ascitic fluid are placed in narrow test tubes (6″ x ½″). This mixture is semifluid in consistency. After the swab has been infected with the gonorrheal discharge it is placed in a tube containing this medium, care being taken that the swab becomes well moistened with it, and is left there. This tube is then placed under the clothing; preferably next to the skin. It is advisable, although not essential, to warm the medium slightly just before introducing the swab. On reaching the laboratory the tubes containing the swabs are placed at once in the incubator. The plates, which should be slightly warmed, may be seeded then or the tubes may be left there for 3 or 4 hours before this is done. Within this period the gonococci apparently do not die out at all; in fact, they may begin to increase in numbers. The plating, however, should not be delayed so long that the contaminating bacteria have an opportunity to overgrow the gonococci. Even at room temperature gonococci in pus deposited on the surface of an ascitic semisolid agar tube and kept in a dimly lighted place have remained viable for a surprisingly long time. In 2 such experiments living gonococci were found after 48 hours in pus left under such conditions, although their numbers were reduced to a fraction of 1% of that originally present. After 3 days all had died. These specimens of pus were obtained from cases of acute urethritis in males and contained large numbers of gonococci. The tests offer a fair criterion of the probable maximal infectious period of such gonorrheal discharges, when kept moist at ordinary temperatures and removed from contact with strong light.

In taking specimens from cases of urethritis in males it was not found necessary to clean or treat the meatus in any way, especially if the discharge was taken up with a platinum loop. Pus from joint

cases should be distributed among 3 or 4 tubes of the semifluid ascitic agar, described in the foregoing paragraph, and plates seeded from them at once and after incubation for 24 to 48 hours.

In seeding plates the swabs are applied to only about one-fourth of the surface area of the medium. The swab should be rolled so that all parts come in contact with the medium. With a platinum loop the other three-fourths of the plate surface are now seeded by streaking from the part to which the swab was applied. In that way a proper distribution of colonies is likely to be obtained on some part of the plate. Before a second plate is seeded the swab should be reintroduced into the tube containing inoculated semifluid ascitic agar and so on for each plate.

In the preparation of plates for gonococcus isolation we have employed both A and B agar mediums, using with each 2 parts of the medium and 1 part of ascitic fluid. As is well known, some samples of ascitic fluid are unsuited for gonococcus culture. The lots used successfully were free from bile and had a specific gravity of 1.010 or higher. As will be explained presently, certain dyes have been used at times in conjunction with these mediums.

Colony Characteristics.—Medium A with ascitic fluid produces a colony which is rather different from that regarded as typical for the gonococcus. These colonies after 48 hours' incubation are only semi-translucent in texture and have a raised, even, or, at most, a slightly indented edge. They stand out prominently from the surface of the medium. The centers are somewhat thickened and exhibit few or numerous light colored granules. The edges are clear and homogeneous. By transmitted light they have a rather characteristic light fawn color with a suggestion of a greenish tinge. In consistency they are somewhat viscid or pasty. On primary isolation the colonies are generally just visible to the naked eye after 24 hours' incubation and are not characteristic in appearance. After 48 hours, however, they may attain a diameter of 1 to 3 mm. and are easily identified. They often continue to grow for about 10 days when they may reach a diameter of 8 mm. The older colonies have heaped up centers with thin spreading edges. One of the advantages of this medium is the unusual retention of viability even in the primary growth; successful replants have been made from such colonies up to 10 days. The gonococcus colonies are also distinctive in appearance and after a little experience are hardly to be mistaken.

The medium B with its growth accessory element may be used to advantage in conjunction with medium A. This plate medium may bring to development over 10 times as many gonococcus colonies as medium A. The colonies also develop more rapidly and may be visible in 18 hours. After 24 hours' incubation they frequently attain a diameter of about 1 mm. These colonies are colorless and translucent, showing a light smoky tinge by transmitted light. They may show heaped up centers or a flat surface. The edge may be very thin and slightly crenated or may be raised, well defined and smooth. The well isolated colonies exhibit the latter appearance and under low magnification show numerous light colored crumbs occupying the entire colony except the outer periphery. Whereas fishings from the A medium plates may be delayed for 3 or 4 days, the gonococcus-like colonies on the B plates should be fished by the second day as the rapid growth seems to be correlated with speedy disintegration. Among other organisms forming a colony similar to the one described in the foregoing, we have noted a gram-postive diplococcus and a small gram-negative coccoid bacillus.

Fishing of Colonies.—Fishings of colonies for the most part have been made into the ascitic semisolid medium (C). The growth on the surface of this medium is so characteristic as to be almost diagnostic in itself. The fished colony is seeded on and by short stabs all over the surface of this medium, which has the advantage of being not only favorable for the primary growth but also for the prolonged viability of the gonococcus. Of such original plants from the plates of 6 cases, the tubes of which had been sealed with paraffin, all were found alive after 50 days at 36 C. and 3 of them after 80 to 90 days. With this medium, then, there is no danger of losing a strain on first isolation and no need of making such frequent replants as has been considered necessary heretofore. It would seem entirely probable that this medium would prove as well adapted for fishing meningococcus colonies. After the first generation of the gonococcus on this ascitic semisolid medium, it is generally possible to transplant successfully to the medium without ascitic fluid; in a number of cases fishings have been made directly to the semisolid medium unenriched with ascitic fluid and growth obtained. Most strains, however, thrive only when the fishing is made to the enriched medium.

Selective Dyes.—Certain dyes, especially gentian violet and brilliant green, have proved so useful in the preparation of selective mediums for

various bacterial types that it was hoped some one might be found of service in the isolation of the gonococcus. A considerable number of dyes were tested and of these, iodin-green and methyl violet gave the more promising results. In most instances the test material consisted of swabbings from mild chronic vulvovaginitis cases in children. Frequently in such cases other types of bacteria are so numerous that isolation of pure cultures of gonococcus is a matter of great difficulty. In the experiments with iodin-green (Grübler), the dye was diluted 1 : 2,000 or 1 : 3,000 and 0.5 to 1 c c added to 15 c c of the ascitic agar medium A. It was observed that this dye, at the dilution used, exercised its inhibitory effect only when glycerol was present in the medium. Under these conditions the dye tends to suppress some types of gram-positive cocci—not streptococci—and certain diphtheroids. Colonies of staphylococci and diphtheroids which succeed in developing on the plates are tinted green, whereas the gonococcus colonies are either uncolored or show only a faint greenish tinge when viewed against a dark background. This dye, in the dilutions used, seemed actually to stimulate the growth of gonococcus as these colonies tend to become larger on plates containing the dye than on the control plates. In fact, the use of this dye in connection with this plating medium was finally adopted more on this account than because of its inhibitory effect on other bacteria, which, indeed, is rather slight.

Of the triphenylmethane dyes, gentian violet and methyl violet were the only ones tested extensively. Gentian violet suppresses effectively the great majority of gram-positive bacteria, but unfortunately gonococcus, although a gram-negative organism, is also highly susceptible to its toxic action. The strongest dilution which could be used with any degree of success was a final one of 1 : 1,200,000 (1 c c of a 1 : 80,000 dye dilution in 15 c c of medium B). Although most gonococcus strains exhibit a slightly greater tolerance to the bacteriostatic action of gentian violet than do the majority of gram-positive cocci and diphtheroids, the margin of difference is so slight that the use of this dye as a selective agent offers slight promise of success. Occasionally, when using gentian violet medium in making cultures from gonorrheal discharge, we have obtained a plate showing a nearly pure culture of gonococcus with a marked suppression of the gram-positive organisms in the specimen, but comparison with the control plate would indicate that only a very small percentage of the viable gonococci in the pus had developed

into colonies. On the gentian violet plates, also, cultures did not become visible to the naked eye until the second or third day of incubation. Cook and Stafford[12] have recently reported on the use of gentian violet and also other triphenylmethane dyes as ingredients of a selective medium for the gonococcus. Gentian violet was used in connection with testicular or chocolate blood testicular agar in a dilution of 1 : 500,000. The results obtained, however, in the application of this medium to clinical diagnosis were not promising.

As is well known, Churchman[24] has advocated the use of gentian violet in the local treatment of cases of purulent arthritis including the gonorrheal type. In discussing the results obtained, he observed that as the gonococcus is a gram-negative organism it might be expected to be relatively unaffected by gentian violet, but he was unable to settle this point experimentally. Our experiments would seem to indicate, however, that the gonococcus exhibits only a slightly lower degree of susceptibility to the bacteriostatic action of gentian violet than do the gram-positive cocci and hence should be classified as a "gentian violet positive organism."

In connection with the isolation of the gonococcus, methyl violet has yielded somewhat better results in our hands than has gentian violet. In our more successful experiments this dye has been diluted to about 1 : 125,000 and 1 c c added to 15 c c of the ascitic agar medium B. A stronger dilution than this tends to be too inhibitory of the development of gonococci. Staphylococci have been quite effectively suppressed as have also some troublesome types of diphtheroids and spore-bearing bacteria. On the other hand, streptococci and gram-negative bacilli are not affected at all. This dye, accordingly, is only to a limited degree selective for the gonococcus, but we have found at times that its presence in the medium has permitted the isolation of this organism when the control plates were covered with growth of other bacterial types. The advantages and limitations in the use of this methyl violet medium may be estimated from the results reported in a following article.[25] As will be observed, it may be used to some advantage in connection with other plating mediums but dependence should not be placed on it alone. This dye has been used only with the ascitic "vitamine" agar B. The optimal dilution should be determined for each

[24] Ibid., 1920, 75, p. 583.
[25] Jour. Infect. Dis., 1922, 31, p. 148.

sample of dye used. Erickson and Albert [9] have recently reported that of various violet and green dyes tested, methyl violet was the most effective for isolation of the gonococcus.

Reaction.—The final reaction of these plating mediums has always been close to P_H 7.2.

CRITERIA FOR THE IDENTIFICATION OF THE GONOCOCCUS

If the material for culture has been obtained from the genito-urinary tract, we have found that the type of colony formation together with morphology and staining reaction is an almost infallible guide to the identification of the gonococcus. As has been mentioned, only one or two types of bacteria from this locality form colonies closely resembling that of the gonococcus and in no case do these bacteria bear a resemblance morphologically to this organism. Cultures from several hundred cases of gonorrhea have never shown any colonies of M. catarrhalis or any other diplococcus morphologically resembling the gonococcus, with its typical picture of a mixture of well-staining, biscuit-form diplococci and swollen, more or less completely autolyzed, irregularly staining cocci and diplococci. Although this is the case, we have not depended on these points alone for identification.

Inability to grow on unenriched medium during the first few generations has been generally accepted as an important diagnostic test. The great majority of gonococcus strains will conform to this requirement, but occasionally we have encountered undoubted gonococcus strains which would grow slowly on ordinary glycerol, beef infusion, peptone agar slants with a reaction of P_H 7.4, on planting from the second or third generation of subcultures. These strains were mostly isolated from vulvovaginitis infections in children. Some eyars ago Wollstein [26] reported that strains from such sources grew readily on plain agar. This capacity, however, may not be considered as a cultural feature differentiating strains causing infantile vulvovaginitis from those concerned in the gonorrhea of adults, for we have isolated from these children's cases strains of a type which was very delicate and difficult to cultivate. No gonococcus strain, of course, will grow at room temperature on even the most favorable type of medium. Serologic tests, such as agglutination and agglutinin absorptions, cannot be depended on as a certain guide to identification, as has been explained elsewhere. That fermentation tests are of great value in the differentiation of the

[26] Jour. Exper. Med., 1907, 9, p. 588.

gram-negative diplococci has been demonstrated by Elser and Huntoon and others. As will be shown presently, our tests with over 80 strains proves that the gonococcus is capable of splitting glucose alone and is thus to be differentiated from its nearest relative, the maltose fermenting meningococcus.

The characteristics distinguishing the gonococcus from other similar organisms may then be summarized as follows: appearance of the colony, reaction to the gram stain and morphology, inability to grow at room temperature and typical fermentation reactions.

FERMENTATION TESTS

Elser and Huntoon,[27] in an extensive series of fermentation tests with different species of gram-negative diplococci capable of a parasitic existence within the human body, found that of the 10 carbohydrates employed in the tests, the gonococcus was the only representative of this group which fermented glucose. In this conclusion they confirmed the earlier finding of Rohe [28] that of glucose, maltose and levulose, the gonococcus splits glucose alone. Within recent years there has been a general agreement that the gonococcus does not ferment maltose and by that fact may be differentiated from the meningococcus. As to the action of gonococcus on galactose, however, there is no such uniformity of opinion. The earlier observers, Dunn and Gordon,[29] Arkwright [30] and Sherman and Ritchie,[31] all report the fermentation of galactose by gonococcus. Elser and Huntoon, however, obtained negative results in tests with 15 strains of this organism. More recently Cole and Lloyd [5] reported that galactose was fermented by the gonococcus but that the acidity was not as great as when glucose was the test sugar. As a probable explanation of the discrepancies in these findings certain experiments of Elser and Huntoon with galactose may well be cited. They found that with intermittent streaming steam sterilization for the customary time periods, galactose and also levulose in a menstruum containing very small amounts of free alkali were likely to be hydrolyzed with the production of acidity, and that this change continued in some instances when the medium was exposed to incubator temperature. However, by using glassware devoid of free alkali and by sterilizing the

[27] Jour. Med. Research, 1909, 20, p. 377.
[28] Centralbl. f. Bakteriol., O., 1908, 46, p. 645.
[29] Brit. Med. Jour., 1905, 2, p. 421.
[30] Jour. Hyg., 1907, 7, p. 145.
[31] Jour. Path. & Bacteriol., 1908, 12, p. 456.

sugars separately through exposure of the distilled water solutions to live steam for 10 minutes, they demonstrated that these hydrolytic changes may be avoided.

As a base for our fermentation tests we have employed a sugar-free broth plus ascitic fluid. Beef infusion was made sugar-free by planting with B. coli and incubating for 24 hours. To the filtrate was added 1% peptone and 0.5% NaCl. After adjusting the reaction to P_H 7.0, the medium was tubed in 5 c c amounts and autoclaved. One c c of ascitic fluid was then added to each tube. The ascitic fluid used had been in cold storage for over 4 months, a period sufficiently long to permit the complete disappearance through hydrolysis of any fermentable carbohydrate which it might have contained. 12% solution of the sugar in distilled water was exposed to flowing steam at 100 C. for 12 minutes and 0.5 c c was added to each tube of the ascitic broth. These tubes of medium were then incubated for 3 days at 37 C. as a test for sterility. The consistent results obtained with levulose, galactose and maltose indicate that these relatively unstable sugars were not injured by the degree and period of heating applied in sterilization.

In these comparative fermentation tests we have used a fluid medium because it permitted a determination of the exact degree of change in reaction through the use of colorimetric methods. As a routine method, however, a solid or semisolid medium to which an indicator has been added is to be preferred as the growth of the gonococcus is much more rapid on such a medium and a reading may be made within 24 to 48 hours. A medium of this type, which has given satisfactory results, is described in a following paragraph. As is indicated in table 4, reaction readings for these gonococcus strains in the various fluid sugar mediums were made after 7 days' incubation. The H-ion concentrations were determined through the use of bromthymol-blue or phenol red and comparisons with their respective scales as the conditions called for.

In this tabulation of fermentations (table 4) the results with 60 gonococcus strains are given in detail. In addition 25 gonococcus strains isolated from cases of mild chronic gonorrhea in women were tested on glucose and maltose, making a total of 85 strains. These strains had been isolated from a great variety of clinical conditions, including acute and chronic urethritis in males, vulvovaginitis in children, arthritis cases, septicemias and cervix uteri infections. They also represented widely separated geographical localities: many parts of this country and such foreign countries as Mexico, England, France, Bel-

TABLE 4

FERMENTATION TESTS WITH 60 GONOCOCCUS STRAINS *

Gonococcus Strains	Glucose P_H	Galactose P_H	Maltose P_H	Levulose P_H
1	6.3	7.1	7.3	7.4
2	6.6	7.2	7.3	7.4
3	6.4	7.0	7.1	7.5
4	6.0	7.1	7.3	7.4
5	6.3	7.2	7.5	7.6
6	6.4	7.2	7.3	7.3
7	6.2	7.2	7.8	7.8
8	6.5	7.2	7.3	7.4
10	6.4	7.0	7.3	
11	6.2	7.2	7.3	7.4
12	6.2	7.4	7.5	7.3
13	6.0	7.0	7.2	
14	6.4	7.5	7.5	7.8
15	6.2	7.4	7.5	7.4
16	6.5	7.0	7.2	7.1
17	6.2	7.5	7.5	7.6
18	6.2	7.3	7.6	7.3
19	6.7	7.5	7.5	8.2
20	6.2	7.0	7.2	7.3
21	6.2	7.1	8.0	7.6
22	6.6	7.1	7.3	7.3
23	6.4	7.6	8.0	7.3
24	6.5	7.1	7.2	7.3
25	6.1	7.2	7.5	7.4
26	6.0	7.3	7.2	
27	6.3	7.1	7.4	7.3
28	6.2	7.1	7.6	7.3
29	6.2	7.5	7.3	7.8
30	6.0	7.1	8.0	
31	6.2	7.8	8.1	
32	6.0	7.2	7.2	
33	6.0	7.1	7.2	
34	6.0	7.6	7.2	
35	6.7	7.4	8.0	
36	7.2	8.0	7.4	
37	6.2	7.2	8.0	
38	6.2	7.2	7.2	
39	6.2	7.3	7.3	
40	6.5	6.9	7.1	
41	6.2	7.2	7.4	
42	6.2	7.2	8.2	
43	6.3	7.1	7.4	
44	6.2	7.3	7.8	
45	6.3	7.3	7.3	
46	6.5	7.2	7.8	
47	6.5	7.2	7.3	
48	6.2	...	7.3	
49	6.2	7.3	7.3	
50	6.2	7.1	7.4	
51	6.4	7.2	8.0	
52	6.0	7.3	7.2	
53	6.2	7.1	7.2	
54	6.2	7.3	7.4	
55	6.2	7.4	7.8	
56	6.5	7.4	7.9	
57	6.3	7.2	7.2	
58	6.5	7.4	7.8	
59	6.4	7.1	7.3	
60	6.7	7.4	7.1	
61	6.2	7.4	7.8	
Control: meningococcus	5.9	7.3	5.8	
Control 1 (sugar mediums uninoculated and incubated)	7.2	7.1	7.2	7.4
Control 2 (medium without sugar inoculated with gonococcus and incubated)	7.2			

* All tests for H-ion concentrations were made after 7 days' incubation.

gium, Germany and Egypt. As may be noted, with one exception these strains all produced a definite acidity in the glucose broth medium. The strain failing to split this sugar was an old stock culture (strain "G," redesignated 36). This strain grew very well in the medium but in repeated tests failed to attack the sugar at all. It would seem likely that we have here an instance of repressed or lost function rather than an exception to the general rule that the gonococcus ferments glucose. The strains differed considerably in the amount of acid produced but this variation was not definitely correlated with either the age of the strain or the amount of growth; some of the strains producing only a slight amount of acid were among the most vigorous growers.

As is indicated in table 4, none of the strains tested fermented galactose, maltose or levulose. The results with galactose are of rather more academic than practical interest as, according to the results of Elser and Huntoon, none of the gram-negative diplococci, which bear a close resemblance to the gonococcus, split this sugar. On the other hand, the uniform absence of action on maltose adds confirmation to the value of this sugar for the differentiation of the gonococcus from the meningococcus. The meningococcus strain, used as a control, acted promptly on this maltose medium, producing a marked degree of acidity in 48 hours. No recent observer has definitely claimed that the gonococcus may ferment maltose. Hermanies [12] recently reported in reference to a large number of gonococcus strains that "practically none of them fermented maltose," the exceptions apparently being 2 strains giving rise to a slight initial acidity which gave way after 48 hours to alkalinity. Wollstein,[26] in 1907, claimed that 10 strains of gonococcus from cases of vulvovaginitis in infants, all fermented maltose. Our tests, however, have indicated that such infantile strains do not differ in their fermentations from strains isolated from adults; 8 such strains (48, 54, 55, 56, 57, 58, 60, 61) gave positive results with glucose and negative results with galactose and maltose.

In the galactose and levulose medium, and to a rather more marked extent in the maltose medium, some degree of alkalinity was produced by the majority of the strains after 7 days' incubation. This degree of alkalinity was quite definitely linked with the vigor of the growth.

Our results have shown definitely that the presence of a sugar fermentable by the gonococcus in a medium does not enhance its growth, for fully as vigorous growth occurred in this fluid medium without any sugar or with the nonfermentable sugars as in the glucose tubes. The

same finding also held true for solid mediums. We can thus confirm the conclusion of Cole and Lloyd [5] that the addition of glucose to mediums prepared for gonococcus culture is not desirable.

A few of our gonococcus strains grew so poorly in this fluid medium that it could not be used for determining their fermentative activities. This medium is also not well adapted for diagnostic tests as the necessary incubation period covers several days. We have found that a sure growth with a definite reading after about 24 hours' incubation may be obtained by using a semisolid agar medium to which brom-thymol-blue has been added as an indicator. This medium is prepared with meat-infusion, sugar-free, 1.5% peptone broth to which ⅔% agar is added. To this semisolid agar, adjusted to P_H 7.0, is added brom-thymol-blue in an amount sufficient to give a fairly deep color. It is tubed in 5 c c amounts, sterilized, and the ascitic fluid and sugars added as advised for the fluid medium. The gonococcus growing on the surface of this unslanted medium causes, within 24 to 48 hours in the presence of glucose, a definite change in color from bluish green to yellow in the medium immediately below the growth. With some strains the change to yellow is evident within 18 hours. In the presence of maltose or other nonfermentable sugar the color either remains unchanged or a bluish tinge develops. This dye indicator does not inhibit the growth of the most delicate strains.

MAINTENANCE OF STOCK STRAINS OF GONOCOCCUS

The maintenance of a large collection of gonococcus strains on ascitic agar or blood agar slants is a troublesome matter and necessitates their being transplanted at intervals of a week or less, in fact some investigators [9] have advised daily replanting of stock cultures. We have found that these difficulties may be in large measure eliminated by the use of the semisolid medium C, which contains a growth accessory principle and is prepared, with slight modifications, according to the method of Huntoon. In using this medium the gonococcus growth is seeded into the upper one-fourth inch or so of the unslanted agar, which has a H-ion concentration of about 6.8. One of the great advantages of this medium is that it is sterilizable, and one may thus avoid the contaminations which are so prone to occur if ascitic fluid or other nonsterilizable albuminous fluids are used; also this medium does not dry out, and hence favors the development of a thick moist growth with most gonococcus strains. With stab cultures into this medium, the

growth is limited to about 4 mm. below the surface, none occurring in the depths of the stab. Huntoon also recommended a semisolid medium of this type for the preservation of stock cultures, reporting a viability period of 3 months for the meningococcus and 2 months for the gonococcus.

The viability factor with this medium has already been discussed from the standpoint of various H-ion concentrations. As was pointed out in that connection, some strains remain viable in this medium for periods ranging from several months to one year; other strains of the more delicate type may not give successful replants in this unenriched medium longer than one month. As a matter of routine, accordingly, the stock strains of the gonococcus have been replanted about every three weeks. Any strain failing to grow could always be recovered by the use of this medium enriched with ascitic fluid (see p. 126). These exceptionally delicate strains exhibited on first isolation the common characteristics of producing an unusually viscid growth with rapid autolysis of the cocci. On prolonged cultivation, however, most of them tended to lose these characteristics and to become much more hardy.

Young growths of gonococcus on this medium placed at room or icebox temperature tended to die out quickly. The limit of viability at both these temperatures ranged from less than 3 days to not more than 6 days. Hermanies, on the other hand, using ascitic agar slants, found his stock strains of gonococcus uniformly viable after 8 to 10 days at room temperature, and some remained so for periods up to several months. Cook and Stafford, using an unenriched testicular agar, obtained a viability period limited to 8 days, regardless of whether the cultures were kept at incubator, room or icebox temperatures. We have found the optimal temperature to be between 36 and 37.5 C., although continued viability is possible with temperatures up to 38.5 C.

SUMMARY

For the optimal growth of the gonococcus the reaction of the medium should be set close to the point of absolute neutrality; between P_H 6.8 and 7.4. The reaction range, however, compatible with growth on a semisolid medium containing a growth accessory factor was found to extend from P_H 5.8 to 8.2.

The relation of viability to the reaction of a medium was studied. A slightly acid reaction was found, on the whole, more favorable than

a slightly alkaline reaction. The remarkable retention of viability for one year was noted in reference to one strain seeded on a semisolid "hormone" agar (Huntoon) with a primary reaction of P_H 6.3.

No better growth was obtained by the use of a medium containing a high concentration of amino acids than when prepared with the specified amount of peptone.

The presence of glucose does not enhance the growth of the gonococcus.

The growth stimulating principle in a medium prepared according to Huntoon's method was found to be slightly impaired by exposure in the autoclave to 120 C. for 5 minutes and seriously injured, but not entirely destroyed, after 30 minutes at that temperature.

Abundant moisture in the air of the incubator is a prime requisite for the optimal growth of the gonococcus, especially on first isolation, but a reduced oxygen tension was not found to be advantageous.

Fermentation tests constitute the most valuable single criterion for the differentiation of gonococci from other similar gram-negative diplococci. No one of 86 gonococcus strains tested split maltose, and all but one fermented glucose. None of the strains tested on levulose and galactose split these sugars. A sugarfree, semisolid, ascitic agar medium, with bromthymol-blue as an indicator, has proved satisfactory as a base for fermentation tests. Differential readings may be made after 18 to 24 hours' incubation.

A semisolid, sterilizable medium with a growth accessory factor (Huntoon formula) was found admirably adapted for carrying a large collection of gonococcus strains. Replantings have not been necessary oftener than once in three or four weeks.

Two plating mediums are described which have been found serviceable in the isolation of the gonococcus. One of these mediums is made in some degree selective by the incorporation of a dye, iodin-green. For the best results the reaction is of prime importance; the final H-ion concentration should be about 7.2. A method of collection of gonorrheal pus specimens for culture is described.